FORM PTO-1390 DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV 5-93)		ATTORNEY'S DOCKET NO. **100096.401**
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) **09/202463** **Unknown**
INTERNATIONAL APPLICATION NO. **PCT/SE97/01164**	INTERNATIONAL FILING DATE **27 June 1997 (27.06.97)**	PRIORITY DATE CLAIMED **5 July 1996 (05.07.96)**

TITLE OF INVENTION
METHODS FOR DETERMINING THE PRESENCE OF BRAIN PROTEIN S-100

APPLICANT(S) FOR DO/EO/US
BRUNDELL, Jan; NYBERG, Lena

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith (required only if not transmitted by the International bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

    Postcard and check for filing fees.

    Applicant hereby claims priority from **Swedish** Application No. **9602677-8** filed **5 July 1996**.

EXPRESS MAIL MAILING LABEL

NUMBER **EM149491550US**

DATE OF DEPOSIT **14 December 1998**

I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C F R. 1 10 on the date indicated above and is addressed to Assistant Commissioner for Patents, Washington D.C 20231-0001.

Amy L Edwards

Signature

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) **Unknown**	INTERNATIONAL APPLICATION NO. **PCT/SE97/01164**	ATTORNEY'S DOCKET NUMBER **100096.401**

17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO ..... $ 840.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $ 670.00

No international preliminary examination fee paid to USPTO (cu CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... $ 760.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... $ 96.00

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$1906.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	18 - 20 =	0	x $ 18.00	$0.00	
Independent Claims	2 - 3 =	0	x $ 78.00	$0.00	
Multiple dependent claim(s) (if applicable)			+ $260.00	$260.00	
TOTAL OF ABOVE CALCULATIONS =				$390.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (NOTE: 37 CFR 1.9, 1.27, 1.28)				$0.00	
SUBTOTAL =				$2296.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$0.00	
TOTAL NATIONAL FEE =				$2296.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property) +				$0.00	
TOTAL FEES ENCLOSED =				$2296.00	
				Amount to be refunded:	$.00
				charged	$.00

a. ☒ A check in the amount of $2296.00 cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **19-1090**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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NAME

31,392
REGISTRATION NUMBER

Inventor(s): JAN BRUNDELL AND LENA NYBERG, . . .

Appln. No.: 0 / _____ or Pate[redacted]o.: _____

Filed: _____ or Issued: _____

Title: Methods for determining the presence of brain protein S-100

(Atty. Dkt. (/ _____)

M# / Client Ref.

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(d) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[ ] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN AB Sangtec Medical

ADDRESS OF CONCERN Box 20045, S-161 02 Bromma, Sweden

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled: Methods for determining the presence of brain protein S-100

by inventors(s) Jan Brundell and Lena Nyberg described in

X ->[ ] the Specification filed herewith,
ene ->[ ] Application No. 0 / _____, filed _____.
box ->[ ] Patent No. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each small entity individual, concern or organization having rights to the invention is listed in (A) and (B) below and no rights to the invention are held by any person, other than the inventor, who could not qualify under 37 CFR 1.9(c) as an independent inventor if that person had made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

(A) FULL NAME of assignee/licensee/grantee/conveyee* _____

ADDRESS _____

proper box: [ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

(B) FULL NAME of assignee/licensee/grantee/conveyee* _____

ADDRESS _____

(proper box: [ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

*NOTE: Separate verified statement is required from each person, concern or organization named in (A) and (B) above having rights to the invention, averring to his/her/its status as a small entity. (37 CFR 1.27)

I acknowledge the duty to file, in this case, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Hans Henriksnäs

TITLE OF PERSON OTHER THAN OWNER Managing Director

ADDRESS OF PERSON SIGNING Box 20045, S-161 02 Bromma, Sweden

SIGNATURE Hans Henriksnäs DATE 18 December 1998

METHODS FOR DETERMINING THE PRESENCE OF BRAIN PROTEIN S-100

The present invention relates methods for diagnosis and follow-up of patients with cerebral dysfunction as well as melanoma cancer, by determining the presence of the brain protein S-100. The invention also relates to peptides comprising useful antigenic determinants from S-100 as well as monoclonal antibodies binding to these peptides.

As is known, the nervous system contains a number of proteins unique to its various cellular elements. The cellular disruption of nervous tissue and cells of neural origin, by any pathogenic process, trauma or by neurological diseases, results in the release of normal soluble endogenous cytoplasmic proteins into the cerebral extracellular fluid and ultimately to other body fluids including the cerebrospinal fluid (CSF) and blood (serum and plasma). Examples of representative soluble small molecule weight proteins of this type can be found in the S100 protein family. A review of this family can be found in Zimmer et al., Brain Research Bulletin, Vol. 37, pp 417-429, 1995.

Following disruption of cell membranes, these proteins are released into the extracellular fluid in accordance with a time course and in quantities relative to the pathogenesis of the disease process or the extent of the brain tissue damages. The proteins diffuse into the CSF and then the blood or directly into the blood. The above mentioned cell membrane disruption is reflected by the blood plasmaor serum levels of one or more of these antigens and markers. These protein antigens have the advantage of being stable and specific, not only for the brain, but for the cellular components in the brain. By following the relative release of the various nervous system protein antigens, it is possible to deduce the kind of destructive process occuring in the course of neurological diseases and/or the extent of possible brain tissue damages. Information of this type permits the diagnosis, evaluation of severity and rate of progression of the above mentioned dieases and damages.

It is previously known to determine the amount of S-100 polypeptides in a clinical sample. US-A-4 654 313 discloses a radioimmunological assay method for S-100

protein. The patent document does neither mention anything about different kinds of S100-polypeptides nor about on which epitopes the assay method is based. The detection limit is declared to be 0.20 ng/ml but concentrations between 1.5 and 2.5 ng/ml is required in order to have less than 10% false positives. This concentration is rather high. Moreover, in some countries it is not permitted to use radioactive methods in clinical assays.

It is also known to determine S-100 polypeptides by using ELISA-related methods. GB-A-2 109 931 discloses a solid-phase immunoanalysis method comprising the use of enzyme-labelled antigens and particles coated with protein A on which antibodies are bound. S-100 proteins are only mentioned in claim 8 and nothing is revealed about the sensitivity of the method.

JP-A-6/109 734 describes a method suitable for analysing S-100 polypeptides, using a first polyclonal antibody fixed to magnetic particles, and a second labelled polyclonal antibody. The method requires two different enzymes, namely horseradish peroxidase and alkaline phosphatase, and it comprises at least ten consecutive steps.The minimum detection limit is stated to be 0.02 ng/ml for cerebrospinal fluid and 0.06 ng/ml for bovine brain.

The complexity of clinical samples is often a serious problem. An assay method may give excellent results with artificial samples in the laboratory but quite a number of unreliable results might be obtained when the method is tested under clinical conditions. When it comes to immunological assays the problems are often caused by an improper selection of antigenic determinants. One antibody in an assay comprising the use of two different antibodies, may be a hindrance to the other antibody when bound to the antigen to be determined. An improper selection of epitope for an antibody involved in the detection process may result in that the detection goup is completely or partially embedded in a protein complex and not available for detection. Different proteins present in the sample might interfere. Moreover, a method comprising many consecutive steps may give uncertain results for complex clinical samples, as the interference possibilities increse with the number of steps and added extra components.

There is always a need for improvment of methods for analysing substances of medical intrest in clinical samples. An ideal clinical assay method should be quick, accurate and possible to perform with all types of clinical samples without degeneration of the accuracy for certain types of specimen. It should also require a minimum of extra components. This applies to determination of S-100 polypeptides as well as other substances of medical interest.

Summary of the invention

Now it has turned out that by using antibodies directed to epitopes in the region from ser1 to asn38 and from thr82 to glu93 of the amino acid sequence of human S-100β polypeptide, an improved clinical assay method for determining S-100 polypeptides and particularly the β subunit or isoform thereof is obtained. Hence, the main object of the present invention is an assay method using monoclonal antibodies directed to these epitopes. Another object of the present invention relates to short peptides having sequences corresponding to parts of the amino acid sequence of the human S-100β polypeptide from ser1 to asn38 and from thr82 to glu93. Yet another object of the present invention relates to analytical kits for carrying out the assay methods.

Detailed description of the invention

As already mentioned above it is often very difficult to outline methods for analysing clinical samples. It is neccesary that the method has a high sensitivity and gives accurate results. It is also very important that known and unknown constituents of the sample other than the analyte do not influence the results. The present invention relates to an immunological assay method for determining the presence and/or content of human S-100 polypeptide based upon a selection of suitable S-100 epitopes and corresponding antibodies which fulfil the above mentioned requirements.

It has turned out that the selected epitope combinations provides tests and test kits where:

1. a high sensitivity is achieved;
2. the antibodies of the kit binds equally strong to the internal standard as to the analyte in the clinical sample;
3. the epitopes are chosen in such a way that the different antibodies do not interfere with each other when they bind to the analyte, i.e. that the epitopes are situated sufficiently distant from each other.

The epitopes of the present invention are all comprised in the human S-100β polypeptide. Epitopes present within the amino acid sequences:
SELEKAMVALIDVFHQYSGREGDKHKLKKSELKELINN (SEQ. ID. NO. 2)
and
TACHEFFEHE (SEQ.ID.NO. 3)
are preferred. Particularly preferred are epitopes comprised within the peptide AMVALIDVFHQYSGREGDKHKLKKSELKELINN (SEQ. ID. NO. 4) and especially within the peptides REGDKHKLKKSELKEL (SEQ. ID. NO. 5) and EFFEHE (SEQ. ID. NO. 6).

The disclosed epitopes are, among all, used to construct peptides for inducing the formation of suitable antibodies on which the claimed assay method is based. These peptides mostly consist of up to 38 amino acids. The whole amino acid sequence of a peptide according to the present invention is derived from human S-100β. These peptides may comprise variants wherein the original amino acid sequence is modified or altered by insertion, addition, substitution, inversion or deletion which preferably show at least 90% homology with the sequence of SEQ. ID. NO. 2 and SEQ.ID.NO.3 and retain essentially the same immunological properties. The peptides may also comprise multiples of certain epitopes, and in this case their sequence length may exceed 38 amino acids.

By the expression "sub-fragment" is meant a polypeptide sequence having a length of at least 6 amino acids.

The epitopes can also be used to construct fusion peptides comprising at least two distinct epitopes which, among all, can be used as internal standard in immunoassays.

Abbreviations

The following abbreviations are used:

S100	-S100β
RT	-Room Temperature
BSA	-Bovine Serum Albumin
Mab(s)	-Monoclonal antibody(ies)
kD	-kiloDalton
ECL	-Enhanced Chemiluminescent Assay
CBB	-Commassie Brilliant Blue
LIA	-Luminometric Immuno Assay
IRMA	-Immuno Radio Metric Assay
ELISA	-Enzyme Linked ImmunoSorbent Assay
SDS-PAGE	-SodiumDodecylSulfate - PolyAcrylamideGelElectrophoresis
PBS	-Phosphate Buffered Saline
RLU	-Relative Light Units
NHS	-N-HydroxySuccinimide
EDC	-N-ethyl-N'-(dimethylaminopropyl)-carbodiimide
RAMFc	-RabbitAnti-MouseFc antibody
EDTA	-EtylenDiamineTetraAcetic acid
NaCl	-Sodium Chloride
NaN_3	-Sodium azide
iv.	-intravenously
aa	-amino acid

ng	-nanogram
ml	-millilitre
mg	-milligram
HRP	-HorseRadish Peroxidase
h	-hour(s)
min	-minute(s)
sec	-second(s)

Experimental details common to all test procedures

The peptides were prepared by the methods disclosed in Merrifield (1963), J. Am. Chem. Soc., vol. 85, p 2149; Gutte et al.(1971), J. Biol Chem vol. 246, p. 1922; and Carpino et al. (1970), J Am Chem Soc vol. 92, p. 5748.

The monoclonal antibodies were prepared by the method according to Köhler et al.(1975), Nature vol. 256, p. 495; and Harlow et al.(1988), Antibodies. A Laboratory Manual, Cold Spring Harbor, p. 139.

Antigen and Standard preparations

Procedure for preparation and purification of S100 antigen prior to immunisation of Balb/c mice was according to Moore (Biochim. Biophys. Res. Comm. 1965, 19: 739 - 744) with a slight modification according to Haglid & Stavrou (J. Neurochem. 1973, 20:1523-1532). Briefly, bovine brain was homogenised in Tris buffer, pH 7.2. The homogenate was centrifuged at 10.000 r.p.m. and the clear supernatant was used for further purification by ammonium sulphate precipitation. The fraction still soluble after saturation by ammonium sulphate was dialysed and purified by separation on a Sephadex G150 Sepharose (Pharmacia Biotech AB, Uppsala Sweden)chromatographic column followed by separation on a DEAE-sephadex (ionic exchange) column (Pharmacia Biotech AB, Uppsala Sweden). The fraction eluted by 0.3 - 0.4 M NaCl was collected, desalted, lyophilised and used for further experiments.

Hybridoma construction.

Balb/c mice were immunised with purified S100ββ intraperitonially in Freund's complete adjuvant and were given booster iv. injection 6 weeks later during 3 consecutive days. The spleen was removed on the fourth day after last injection and prepared for fusion. The myeloma cell line Sp2/0-Ag14 was used for fusion of Balb/c spleen cells.

Antibody purification and subclass determination.

Monoclonal antibodies were identified, extracted and purified from hybridoma supernatant according to Harlow & Lane Eds. in ANTIBODIES A LABORATORY MANUAL, Cold Spring Harbour Laboratory Press, New York 298-299 & 311. Briefly, positive hybridoma clones carrying supernatant with specific antibodies were identified using ELISA with microtitreplate wells coated with S100ββ. Immunoglobulins were precipitated using saturated ammoniumsulphate and dialysed against 1.5 M Glycine, 3 M NaCl, pH 8.9. Dialysed material were affinity chromatography purified on an protein-A Sepharose (Pharmacia Biotech AB, Uppsala Sweden) column. Fractions were neutralised by addition of small volumes of 1M Tris pH 8.0.

Epitope mapping

S100β (monomer) epitopes for respective antibody was investigated by use of a synthetic peptide library. Peptides were linked to nitro-cellulose filter membrane via an amide link, according to the manufacturer (Research Genetics', USA) and covers all ninety-one aa in the protein. In total the library consisted of thirty-one, all except one being ten aa-residues long synthetic peptides. Each peptide was consecutively shifted three aa towards the -COOH terminal end of the protein. Positive antibody-binding was indicated by the use of a second anti-mouse antibody conjugated with HRP and detected using an ECL assay (Amersham, UK).

Results:

Two binding sequences were found

Epitope 1

AMVALIDVFHQYSGREGDKHKLKKSELKELINN (residues 6-38)(SEQ. ID. NO.4)

and

Epitope 2

EFFEHE (residues No 86-91) (SEQ. ID. NO. 6)

Antibody reactivity

Purified antibodies reacting with the epitopes were checked for reactivity and affinity using the BIAcore™ system (Pharmacia Biosensor AB, Uppsala Sweden). Briefly, in order to test the specificity of the antibodies, the RAMFc was immobilised onto the sensor chip CM5 NHS-ester activated surface, according to standard procedure. to provide approximately 600 RLU. Then each Mab was bound to the RAMFc surface to approximately 300RLU. followed by the S100αα and the S100 standard (consisting of 50% S100αβ and 50% S100ββ) in separate experiments. All reactions were carried out in continuos flow of the phosphate buffer.. The kinetics between antibodies and antigen was done similarly . S100 antigen was added to the chips at 200-450nM for reactivity measurements of the antibody intended for the solid phase and at 1000-1500nM for measurements of the antibody intended for tracers. Kinetics was determined using the BIAcore™ Kinetic evaluation 2.1. software (Pharmacia Biosensor AB, Uppsala Sweden). It can be concluded from the reactivity profile that the antibodies reactive with the epitopes are specific for the β-containing forms of S100 and not the α-containing form.

Example 1

Development of an immunoluminometric procedure

Tracer antibody was conjugated with luminol. Briefly, ABEI (Sigma, St Louis, Ms) was linked with a diactivated ester (Etylenglykolbis-succimidyl succinat, EGS). The ABEI-EGS-conjugate was next mixed with monoclonal antiS100-antibody in an approximately 50:5 molar ratio in 100µl of PBS pH 7.4, containing 15% acetonitrile and incubated 1 h at room temperature. The ABEI-conjugated antibody was purified on a Sephacryl®S 300 HR (Pharmacia Biotech AB, Uppsala Sweden) gelfiltration column, and appropriate fractions were pooled and diluted in phosphate-buffer.

Preparation of antibody coated tubes for LIA.

Polystyrene tubes (Greiner, Germany) were incubated overnight at room temperature with 3µg of S100-antibodies in 300 µl of PBS pH 7.5. The tubes were washed with 0.1% Tween20® in PBS. Next, tubes were blocked with a solution containing 0.9%BSA and 4% Saccarose and incubated for 24h. The solution was aspirated and the tubes allowed to dry.

LIA test procedure.

The test was conducted in a two step procedure by incubating 100µl of patient body fluid in antibody coated tubes, or S100 standard with 100 µl of diluent (PBS + 5%BSA) and incubated at room temperature. After washing 200 µl of the luminol-labelled antibody was added and a further 2 h of incubation was performed before measurement. After another washing the luminescence was developed using the LIA-mat starter service kit (Byk-Sangtec, Diezenbach Germany) and immediately measured as integrals over a period of 5 sec in luminometer (Berthold, Germany). In order to convert the obtained light signal into concentration of S100 measurements on patient samples were compared with measurements on solutions with known

concentrations of S100 (standards). The detection limit (zero standard +3 standard deviations) was approximately 0.01 µg/l.

Preparation of a Standard curve

S100B protein was obtained from Medisera, Lund, Sweden, and diluted in PBS + 5% BSA. Dilutions contained: 0.10,0.40, 2.00, 8.00 and 20.00 µg/l, of an S100 preparation consisting of 50 % of the ββ form and 50 % of the αβ form. PBS + 5 % BSA was used as standard 0. Three measurements were carried out for each dilution. The measured results as well as statistical calculations are presented in table 1 below:

Table 1

Concentration	Counts	Average	Calculated conc.	Average
Standard 0	1996		0 µg/l	
	2024		0 µg/l	
	2053		0.0019 µg/l	
		2024		0 µg/l
0.10 µg/l	3142		0.135 µg/l	
	2760		0.0647 µg/l	
	2988		0.105 µg/l	
		2963		0.10 µg/l
0.40 µg/l	5494		0.394 µg/l	
	5620		0.405 µg/l	
	5579		0.401 µg/l	
		5564		0.40 µg/l
2.00 µg/l	21430		2.049 µg/l	
	21028		1.988 µg/l	
	20869		1.966 µg/l	
		21109		2.00 µg/l

Concentration	Counts	Average	Calculated conc.	Average
8.00 µg/l	68389		7.823 µg/l	
	67013		7.677 µg/l	
	74791		8.494 µg/l	
		70064		8.00 µg/l
20.00 µg/l	175560		22.12 µg/l	
	155141		18.54 µg/l	
	161052		19.51 µg/l	
		163918		20.00 µg/l

The lower detection limit was defined as three standard 0 determinations plus 3X the standard deviation value. For this measurement, it was calculated to be 0.006 µg/l.

Clinical determinations of S100 in serum

The S100 concentration was determined in serum from patients receiving heart bypass surgery and being connected to a heart-lung machine. The results are presented in table 2 below:

Table 2

Patient	Counts	Average	Concentration	Average
1	94698		10.61 µg/l	
	98104		10.99 µg/l	
		96401		10.80 µg/l
2	1716		Not detected	
	1478		Not detected	
		1597		Not detected
3	3762		0.23 µg/l	
	3799		0.23 µg/l	
		3780		0.23 µg/l

Patient	Counts	Average	Concentration	Average
4	13158		1.04 µg/l	
	14183		1.15 µg/l	
		13670		1.10 µg/l
5	8788		0.66 µg/l	
	8580		0.64 µg/l	
		8684		0.65 µg/l
6	10301		0.78 µg/l	
	10100		0.77 µg/l	
	10200			0.77 µg/l

Example 2

Development of an ELISA test procedure.

As tracer antibody was used monoclonal antiS100 antibody conjugated with β-galactosidase according to Harlow & Lane Eds. in ANTIBODIES A LABORATORY MANUAL. Cold Spring Harbour Laboratory Press, New York page 351.

Preparation of antibody coated microtiter wells for ELISA.

Microtiterplatewells (Corning, Denmark) were incubated overnight at +4°C with 2.5µg of microtiter wells were finally washed three times with 0.05% Tween20® and air dried before use.

ELISA test procedure.

The ELISA was conducted in a multiple step incubation procedure.
100 µl of 1:1 diluted patient sample or 100µl of S100 standard (0 - 20 µg/ml) was added to the wells.
The plate was incubated for 1.5h at RT under shaking.

The plates were washed three times with 300µl 0.05% Tween20® in PBS.

100 µl of alkaline phosphatase conjugated tracer antibody was added and a further 1.5h of incubation on a shaker was performed.

The wells were then washed three times with 0.05% Tween20® in PBS.

100µl of a 5% o-nitro-phenyl-β-galactoside substrate solution was added and the plates were incubated with substrate for another forty-five minutes and colour is developed.

The colour development was stopped by the addition of 100µl 0.66M Na_2CO_3.

Each well of the plate was read at 405nm in a standard microtiterplate reader. In order to convert the obtained colour signal into concentration of S100, measurements on patient samples were compared with measurements on solutions with known concentrations of S100 (standards). The detection limit (zero standard +3 standard deviations) was approximately 0.2 µg/l.

Result:

Standard (µg/l)	0	0.5	1.5	5	15
A 405	0.088	0.147	0.244	0.675	1.196

Example 3

Development of an immunoradiometric (IRMA) test procedure

IRMA tracer antibody conjugation

A monoclonal antiS100 antibody was conjugated with Iodine using the Chloramine T method according to Greenwood et al. (Biochem. J. 1963, 89:114-123). The specific activity was determined to be 520±80 MBq/mg

Preparation of antibody coated to polystyrene beads

Monoclonal anti S100 antibodies were coupled to polystyrene beads by the Glutaraldehyde coupling method according to Harlow & Lane Eds. in ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbour Laboratory Press, New York, 533 & 536-537. Final blocking was by 1% BSA, 0.1% NaN_3 in PBS pH7.5.

IRMA test procedure.

100µl of patient sample or standard was added to polystyrene tubes together with 100µl PBS diluent. One polystyrene coated bead was added to each tube followed by incubation for 1 h at RT on a shaker. Next the beads were washed once with 2ml of demineralised water and 200µl of I-125 labelled tracer antibody was added and the tubes were incubated a further 2h on a shaker. After washing the radioactive signal on the bead was measured in a standard γ-counter. In order to convert the obtained radioactive signal into concentration of S100, measurements on patient samples were compared with measurements on solutions with known concentrations of S100 (standards). The detection limit (zero standard +3 standard deviations) was approximately 0.1 µg/l.

Example 4

Use of IRMA test procedure for assay of S100 in serum from melanoma patients

The S100 based test procedure was applied on clinical questions relating to melanoma. Blood samples from patients with melanoma of various stages of cancer progression were collected in serum collecting tubes. Samples were then frozen and treated according to the test procedure described above in Example 3.

Results:

Relationship to staging.

Clinical Stage I vs Clinical Stage II. In a study of 577 patients the geometric mean for Stage I was found to be 0.12 µg/l and for Stage II the geometric mean was found to be 0.33 µg/l.

p-value < 0.001.

Example 5

Use of IRMA test procedure for assay of S100 in serum from melanoma patients

The S100 based test procedure was applied on clinical questions relating to melanoma. Blood samples from patients with melanoma of various stages of cancer progression were collected in serum collecting tubes. Samples were then frozen and treated according to the test procedure described above in Example 3.

Results:

Relationship to survival

Clinical Stage I vs Clinical Stage II and III. In a study with respect to survival performed on 643 patients the relative hazard and 95% confidence interval was calculated. The relative hazard was found to be 12.3 and the confidence interval 5.6-27.2 with a p-value of <0.001

Example 6

Use of the S100 LIA-method for evaluation of the influence of extra corporal circulation equipment on the brain

The S100 based test procedure in Example 1 was applied on monitoring cerebral injury following extra corporeal circulation (ECC). Blood samples from patients undergoing extra corporeal circulation were collected in serum tubes and treated according " Test procedure". Results

	Before start of ECC	End of ECC	1 day after surgery	2 days after surgery
S100 levels µg/l	0	1,67	0,21	0,13

In this group of patients the level of S100 in serum was elevated for at least 2 days after surgery.

Uncomplicated cases should return to normal levels within the first 24 hours (Ref P. Johnson et al. J. Cardiothor .Vasc. Anaesthesia, 9:6 (1995) 694-99).

Example 7

Use of LIA test procedure for assay of S100 in serum from melanoma patients

The S100 based test procedure was applied on clinical questions relating to melanoma. Blood samples from patients with melanoma of various stages of cancer progression and blood donors were collected in serum collecting tubes. Samples were frozen and treated according to the test procedure described above in Example 1.

Result: Of 136 patients with various stages of melanoma 25 had a level of S100 below 0.08 and of 100 blood donors tested on the same occasion 7 had a level equal to or above 0.08 µg/l.

Example 8

The reliability of both the test and the S100β polypeptide marker per se when diagnosing melanoma were investigated. On 252 patients with melanoma. serum was drawn before treatment was started and determination of the level of S100β polypeptide was performed by the assay method disclosed in example 1. When a cut-off value of 0.16 µg/l was used. the medium survival time of patients having a S100β concentration above the cut-off value was 7 months. whereas the medium

survival time was more than 120 months for patients having a S100β concentration below the cut-off value.

In a patient diagnosed with malignant melanoma, considered to show no evidence of disease and monitored by the immunoradiometric assay method as disclosed in example 3, elevated levels of S100β were recorded 2 months prior to the appearance of skin metastases and 6 months before metastases in organs were found.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Methods for determining brain antigens

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Glu Leu Glu Lys Ala Val Val Ala Leu Ile Asp Val Phe His Gln
1 5 10 15

Tyr Ser Gly Arg Glu Gly Asp Lys His Lys Leu Lys Lys Ser Glu Leu
20 25 30

Lys Glu Leu Ile Asn Asn Glu Leu Ser His Phe Leu Glu Glu Ile Lys
35 40 45

Glu Gln Glu Val Val Asp Lys Val Asn Glu Thr Leu Asp Ser Asp Gly
50 55 60

Asp Gly Glu Cys Asp Phe Gln Glu Phe Met Ala Phe Val Ala Met Ile
65 70 75 80

Thr Thr Ala Cys His Glu Phe Phe Glu His Glu
85 90

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Glu Leu Glu Lys Ala Met Val Ala Leu Ile Asp Val Phe His Gln
1 5 10 15

Tyr Ser Gly Arg Glu Gly Asp Lys His Lys Leu Lys Lys Ser Glu Leu
20 25 30

Lys Glu Leu Ile Asn Asn
35

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Thr Ala Cys His Glu Phe Phe Glu His Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Met Val Ala Leu Ile Asp Val Phe His Gln Tyr Ser Gly Arg Glu
1 5 10 15

Gly Asp Lys His Lys Leu Lys Lys Ser Glu Leu Lys Glu Leu Ile Asn
20 25 30

Asn

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Arg Glu Gly Asp Lys His Lys Leu Lys Lys Ser Glu Leu Lys Glu Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6.

Glu Phe Phe Glu His Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 7.

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Asp Lys His Lys Leu Lys Lys Ser Glu Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Lys Leu Lys Lys Ser Glu Leu Lys Glu Leu
1 5 10

Claims

1. A peptide consisting of at least one sub-fragment of the human S-100β polypeptide comprising from 6 to 38 amino acids, where said sub-fragments show at least 90% homology with the sequence

SELEKAMVALIDVFHQYSGREGDKHKLKKSELKELINN (SEQ. ID. NO. 2)

and/or the amino acid sequence

TACHEFFEHE (SEQ. ID. NO. 3)

and retain essentially the same immunological properties.

2. A peptide according to claim 1 **characterized** in that the sub-fragments are derived from the amino acid sequence:

SELEKAMVALIDVFHQYSGREGDKHKLKKSELKELINN (SEQ. ID. NO. 2).

3. A peptide according to claim 2, which is

REGDKHKLKK (SEQ. ID. NO. 5);
DKHKLKKSEL (SEQ. ID. NO. 7); or
KLKKSELKEL (SEQ. ID. NO. 8).

4. A peptide according to claim 1, **characterized** in that the sub-fragments are derived from the amino acid sequence:

TACHEFFEHE (SEQ. ID. NO. 3).

5. A peptide according to claim 4, which is

EFFEHE (SEQ. ID. NO. 6).

6. A peptide according to claim 1, **characterized** in that it consists of at least one sub-fragment derived from the sequence according to SEQ. ID. NO. 2 and at least one sub-fragment derived from the sequence according to SEQ. ID. NO. 3.

7. A monoclonal antibody or a fragment of such an antibody specifically binding a peptide according to anyone of the preceeding claims.

8. A monoclonal antibody or an antibody fragment according to claim 7, specifically binding a peptide according to claim 2.

9. A monoclonal antibody or an antibody fragment according to claim 7, specifically binding a peptide according to claim 4.

10. Use of a monoclonal antibody or an antibody fragment according to anyone of claims 7-9 in immunological assay methods.

11. Use of a peptide according to anyone of claims 1-6 for eliciting antibodies.

12. Use of a peptide according to anyone of claims 1 - 6 in immunological assay methods.

13. A method of determining the presence of human S-100β polypeptide in a sample comprising the steps of:

letting the sample to be analyzed immunologically react with a first monoclonal antibody according to claim 8, said first antibody being coupled to a carrier;

letting the sample immunologically react with a second monoclonal antibody according to claim 9, said second monoclonal antibody being provided with detection means;

Washing; and

detecting the amount of S-100β polypeptide in the sample.

14. A method according to claim 13 where the detection means is a group having the ability of emitting luminescence.

15. A method according to claim 14, where the carrier is a magnetic particle.

16. A kit for determining the presence of human S-100β polypeptide in a sample, comprising a peptide according to anyone of claims 1 - 6 and/or an antibody according to anyone of claims 7 - 9.

17. A kit according to claim 16 comprising a first monoclonal antibody according to claim 8 and a second monoclonal antibody according to claim 9, said first monoclonal antibody being coupled to a carrier and said second monoclonal antibody being provided with a detection means.

18. A kit according to claim 17, wherein said carrier is a magnetic particle and said detection means is a group having the ability of emitting luminescence, such as luminol.

DECLARATION AND POWER OF ATTORNEY

As the below-named inventors, we declare that:

Our residences, post office addresses, and citizenships are as stated below under our names.

We believe we are the original, first, and joint inventors of the invention entitled "Methods for Determining the Presence of Brain Protein S-100," which is described and claimed in the foregoing specification and for which a patent is sought.

We have reviewed and understand the contents of the foregoing specification, including the claims, as amended by any amendment specifically referred to herein (if any).

We acknowledge our duty to disclose information of which we are aware which is material to the patentability and examination of this application in accordance with 37 C.F.R. § 1.56(a).

We hereby claim foreign priority benefits under 35 U.S.C. § 119 of the foreign patent application or of any PCT international application designating at least one country other than the United States of America listed below and have also identified below any foreign patent application patent or any PCT international application(s) designating at least one country other than the United States of America filed by us on the same subject matter having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:			
COUNTRY	APPLICATION NUMBER	DATE OF FILING	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/SE97/01164	05 JUN 96	YES

We hereby appoint RICHARD W. SEED, Reg. No. 16,557; ROBERT J. BAYNHAM, Reg. No. 22,846; EDWARD W. BULCHIS, Reg. No. 26,847; GEORGE C. RONDEAU, JR., Reg. No. 28,893; DAVID H. DEITS, Reg. No. 28,066; WILLIAM O. FERRON, JR., Reg. No. 30,633; PAUL T. MEIKLEJOHN, Reg. No. 26,569; DAVID J. MAKI, Reg. No. 31,392; RICHARD G. SHARKEY, Reg. No. 32,629; DAVID V. CARLSON, Reg. No. 31,153; MAURICE J. PIRIO, Reg. No. 33,273; KARL R. HERMANNS, Reg. No. 33,507; DAVID D. MCMASTERS, Reg. No. 33,963; MICHAEL J. DONOHUE, Reg. No. 35,859; CHRISTOPHER J. DALEY-WATSON, Reg. No. 34,807; STEVEN D. LAWRENZ, Reg. No. 37,376; ROBERT G. WOOLSTON, Reg. No. 37,263; ELLEN M. BIERMAN, Reg. No. 38,079; PAUL T. PARKER, Reg. No. 38,264; JOHN C. STEWART, Reg. No. 40,188; DAVID W. PARKER, Reg. No. 37,414; BRIAN G. BODINE, Reg. No. 40,520; FRANK ABRAMONTE, Reg. No. 38,066; E. RUSSELL TARLETON,

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We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

1-00

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